

Original Article

Inflammatory cytokine profiles of serum and cerebrospinal fluid in Chinese children with hand, foot and mouth disease

Ying Hua¹, Yanping Wang¹, Weijuan Gong^{2,3,4,5,6}

¹Department of Neurology, Wuxi Children's Hospital, Jiangsu Province, China; Departments of ²Immunology, ³Integrated Chinese and Western Medicine, School of Medicine, Yangzhou University, Yangzhou, China; ⁴Department of Gastroenterology, The Affiliated Hospital, Yangzhou University, Yangzhou, China; ⁵Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Jiangsu, China; ⁶Jiangsu Key Laboratory of Zoonosis, Yangzhou University, Jiangsu, China

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Abstract: Objective: Hand, foot and mouth disease (HFMD) is mainly caused by enterovirus 71 (EV71) or coxsackie virus A16 infection. Severe HFMD with encephalitis is a life-threatening disease for children. This study aimed to examine the levels of a variety of inflammatory cytokines in HFMD patients. Methods: Sera of severe or common HFMD patients and cerebrospinal fluids from severe HFMD patients in the acute or recovery phase were collected to analyse 40 cytokines using the Raybiotec Cytokine array. Twenty-six samples of sera or cerebrospinal fluids from HFMD patients were further detected using Milliplex beads. Results: Numerous inflammatory cytokines, such as IL-8, IP-10, and RAENTES, were enhanced in the sera and cerebrospinal fluids of encephalitis-complicated HFMD patients. Serum IL-12p40 and IL-15 levels were higher in severe HFMD patients compared with common patients. Moreover, IL-8 and IP-10 levels in the cerebrospinal fluid were sharply enhanced by 30-fold on average compared with those in the sera. Cerebrospinal fluid IL-8, IP-10, and RANTES levels were significantly enhanced in the acute phase of severe HFMD patients compared with those in the recovery phase. However, no correlation of serum IL-8 or IP-10 level with its cerebrospinal fluid level was observed. Conclusion: An inflammatory cytokine storm occurs both in sera and cerebrospinal fluids during severe HFMD, which is likely a Th1 cell-associated disease. Cerebrospinal fluid IL-8, IP-10 and RANTES levels could be used as potential biomarkers for diagnosing severe HFMD, whereas the enhanced serum levels of IL-12p40 and IL-15 could be used as candidate predictive factors for severe HFMD.

Keywords: Hand, foot and mouth disease (HFMD), IL-8, IP-10, encephalitis, cerebrospinal fluid

Introduction

Hand, foot and mouth disease (HFMD) is a contagious disease caused by enteroviruses, including enterovirus 71 (EV71) and coxsackie virus A16 [1]. HFMD mainly affects young children under 5 years old. Major symptoms of HFMD include fever, rashes and ulcers in the hand, foot and mouth. Moreover, myocarditis, pulmonary edema, aseptic meningitis and other fatal complications may occur in patients with severe infection [2]. Early diagnosis of severe HFMD is particularly important because early intervention can usually reverse the disease development [3]. Thus, investigating several biomarkers is valuable for the early diagnosis of severe HFMD in clinical settings.

Inflammatory cytokines are present in the serum of HFMD patients. Serum IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-13, IL-18, IL-22, IL-23, IL-33, tumour necrosis factor (TNF)- α , granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage inflammatory protein (MIP)-1 β are significantly higher in HFMD patients compared with healthy controls [4, 5]. Serum levels of interleukin (IL)-4, IL-5, IL-22, IL-23, G-CSF and monocyte chemotactic protein (MCP)-1 are enhanced in patients complicated with encephalitis [6]. Furthermore, IP-10, MCP-1, IL-6, IL-8 and G-CSF levels are higher in the cerebrospinal fluid than in the plasma in neurological compromised patients [7].

The Th1/Th2 and Th17/Treg ratios are enhanced in children with HFMD [8, 9]. The

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Table 1. Clinical characteristics of HFMD patients

| Characteristics | Severe n=26 | Common N=26 | P value |
|---|-------------------|-------------------|--------------------|
| EV-71 infection | 65.4 (17/26) | 34.6 (9/26) | 0.027 ^a |
| Median age (month) (range) | 34.00 (7-96) | 33.31 (9-144) | 0.159 |
| Gender (Female/Male) | 9/17 | 11/15 | 0.096 ^a |
| Time of illness at sampling, (day) (range) | 2.92 (0-4) | 3.07 (0-5) | 0.595 |
| Median WBC, (/mm ³) (range) | 11.67 (6.8-17.4) | 9.62 (4.6-15.8) | 0.043 |
| Median neutrophils, (%) (range) | 55.80 (33.1-80.6) | 40.76 (32.4-52.6) | <0.05 |
| Median lymphocytes, (%) (range) | 36.43 (13.7-58.7) | 49.87 (32.2-59.4) | <0.05 |
| Median monocytes, (%) (range) | 6.77 (3.4-12.1) | 8.21 (2.2-11.2) | 0.347 |
| Median platelets, (/mm ³) (range) | 255 (112-425) | 252 (104-424) | 0.887 |
| Median peak fever temperature, (u) (range) | 39.2 (37.9-41) | 39 (37.5-40.3) | 0.39 |
| Fever | 100% (26/26) | 100% (26/26) | >0.05 ^a |
| Oral ulcer | 100% (26/26) | 100% (26/26) | >0.05 ^a |
| Rash | 100% (26/26) | 100% (26/26) | >0.05 ^a |

^aP values were analyzed via X² test; the rest P values were analyzed by Student's t-test. WBC, white blood cells.

increased EV71 epitope-specific Th2-type response may predict poor prognosis for certain HFMD patients [10]. Altered cell subset frequency (imbalance) during infection leads to altered cytokine patterns. Therefore, we aim to systematically analyse the serum and cerebrospinal fluid levels of inflammatory cytokines in patients with either uncomplicated HFMD or severe HFMD complicated with central nervous system (CNS) involvement. Moreover, cytokine level variations in cerebrospinal fluids of HFMD patients at different phases were analysed. The study shows direct data of the cytokine profiles from the serum and cerebrospinal fluid of HFMD patients with different severities and provides an important cue for diagnosing severe HFMD.

Material and methods

Study subjects

All subjects were enrolled in the Children's Hospital of Wuxi City in Jiangsu Province from 2014 to 2015. HFMD patients were diagnosed according to the criteria of Ministry of Health, namely, 'Guide of diagnosis and treatment of the hand, foot and mouth disease (2013 edition)'. According to clinical manifestations, HFMD infections were divided into common and severe groups. Patients in the common group exhibited clinical manifestations of fever, herpes or oral ulcers, and visible rash or herpes in the hands or feet. Patients in the severe group showed nervous system involvements

(headache, vomiting, lethargy and trembling of limbs). This study was approved by the Ethics Committee of the Children's Hospital of Wuxi City. Informed consent regarding the use of serum or cerebrospinal fluid samples was obtained from the guardians of patients.

Serum and cerebrospinal fluid samples

Serum samples were obtained from the common or severe HFMD patients. Sera from 26 non-HFMD patients who underwent minor operations for inguinal hernia or hydrocele were used as controls. Cerebrospinal fluid samples were collected from the severe HFMD patients in both acute and recovery phases. As control, cerebrospinal fluid was obtained from children with non-infectious neurological system disorders, including 12 epilepsy cases, 9 vascular headache cases, 4 febrile seizure cases and one hysteria case. The clinical characteristics of HFMD patients are shown in **Table 1**. Stool samples from all patients were tested for enterovirus 71, coxsackie virus A6 and A16 infection by a real-time RT-PCR assay using a commercial kit. The diagnostic criteria of children in the recovery phase were as follows: normal body temperature, good spirit, disappearance of headache, vomiting, drowsiness and other neurological symptoms.

Cytokine array

Cytokines, chemokines, growth factors and soluble immune receptors in the sera or cere-

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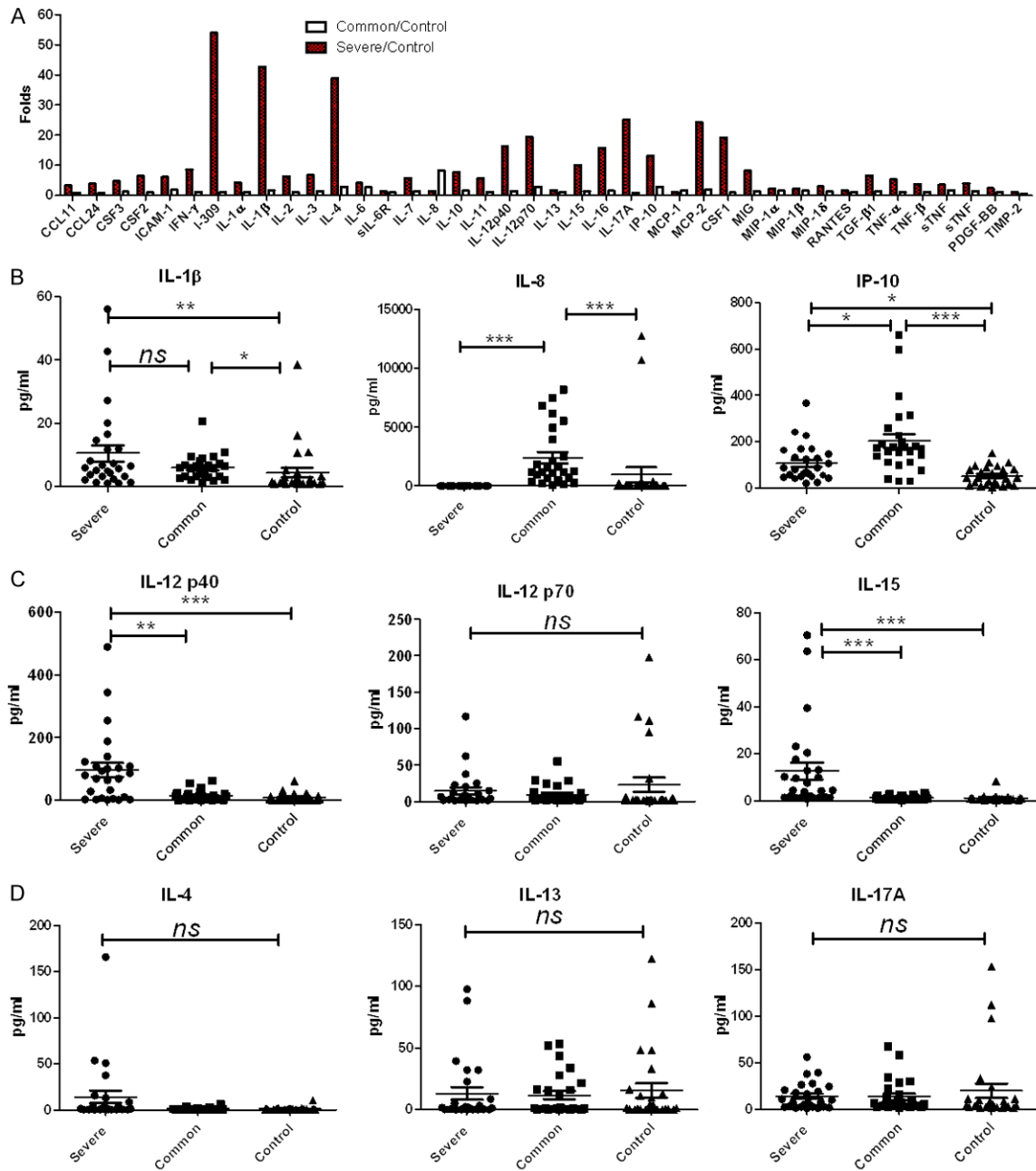


Figure 1. Detection of cytokines in serum from HFMD patients of different severities. (A) Cytokine levels measured using the Raybiotec cytokine antibody array (n = 3). IL-1 β , IL-8 and IP-10 (B) IL-12p40, IL-15 and IL-12p40 (C) IL-4, IL-13 and IL-17A (D) detected using Milliplex beads (n = 26). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

brospinal fluid were examined using the RayBio® Human Inflammation Antibody Array 3 (G-Series) according to the manufacturer's instructions (Norcross, GA, US). The RayBio® G-Series Cytokine Antibody Array is a glass chip that can simultaneously detect multiple cytokine levels of biopsy specimen, and the inter-array Coefficient of Variation (CV) of spot signal intensities is 5 ~10% around. Detection limits

of parameters were in line with the manufacturer instruction [11].

Detection of cytokine by luminex

Serum cytokines (IL-1 β , IL-8, IP-10, IL-12p70, IL-12p40, IL-15, IL-4, IL-13, and IL-17A) and cerebrospinal fluid cytokines (IL-1 β , IL-8, IP-10, IL-12p70, IL-12p40, TNF- α , RANTES, and MIP-

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1 α) were determined with the Luminex detection method, which is based on LiquiChip technology (Milliplex). The detection equipment was the Luminex 200 (Luminex Co. Ltd., USA), according to the kit instruction [12].

Statistical analysis

Continuous variables were expressed as means \pm standard deviation. Significant differences between two or multiple groups were analysed using the group Student's *t* test or one-way ANOVA test. Comparison of cytokine levels between serum and cerebrospinal fluid of HFMD patients was conducted using the paired Student's *t* test. Correlation of serum cytokine levels with cerebrospinal fluid cytokine levels was done using linear regression. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the Graphpad 7.0 software (La Jolla, CA, USA).

Results

Serum cytokine profiles in patients with HFMD of different severity

A total of 40 soluble factors of sera from severe HFMD patients, common HFMD patients and controls ($n = 3$) were preliminary examined using the cytokine array. Folds of the cytokine levels of severe HFMD patients versus controls or common HFMD patients versus controls are shown in **Figure 1A**. Levels of CSF-2, ICAM-1, IFN- γ , I-309, IL-1 β , IL-4, IL-2, IL-3, IL-7, IL-10, IL-11, IL-12p70, IL-12p40, IL-15, IL-16, IL-17A, IP-10, MCP-2, CSF-1, MIG, TGF- β 1, and TNF- α in severe HFMD patients showed more than five-fold higher than those of controls. Only the IL-8 level was more than five-fold higher in common HFMD patients than in the controls. When cytokines levels were compared between severe and common HFMD patients, CCL24, CSF-2, IFN- γ , I-309, IL-1 β , IL-4, IL-2, IL-11, IL-12p70, IL-12p40, IL-15, IL-16, IL-17A, MCP-2, CSF-1, MIG, and TNF- α were upregulated by a minimum of five times. These findings confirmed the storm of inflammatory cytokines in severe HFMD patients.

To confirm the variations of serum cytokine levels in HFMD patients of different severities, we detected IL-1 β , IL-8, IP-10, IL-12p40, IL-12p70, IL-15, IL-4, IL-13, and IL-17A ($n = 26$) using Milliplex beads. IL-1 β , IL-8, and IP-10 levels

were higher in common HFMD patients than in controls. IL-8 and IP-10 levels were significantly lower in severe HFMD patients than in common HFMD patients, whereas no significant change in IL-1 β was observed (**Figure 1B**). Although no significant changes of serum IL-12p40 and IL-15 levels were observed between common HFMD patients and controls, serum IL-12p40 and IL-15 levels were higher in severe HFMD patients than in common HFMD patients and controls (**Figure 1C**). No significant variations were observed in IL-12p70 among the three groups. Th2 cell-derived cytokines (IL-4 and IL-13) and Th17 cell-derived cytokine (IL-17) showed no significant changes among severe HFMD patients, common HFMD patients and controls (**Figure 1D**). In total, the Th1 cell-derived cytokines in the sera were associated with severe HFMD patients.

Cytokine profiles of cerebrospinal fluid of HFMD patients in different phases

The 40 soluble factors in the cerebrospinal fluid of HFMD patients in the acute phase, recovery phase and controls ($n = 4$) were preliminary examined and screened using the cytokine array. Folds of the cytokine levels of HFMD patients in the acute phase versus the recovery phase, as well as HFMD patients in the recovery phase versus controls, are shown in **Figure 2A**. The ICAM-1, IL-3, IL-12p40, IL-12p70, IL-16, MCP-2, RANTES, MIP-1 β , and PDGF-BB levels in the acute phase were five-fold higher than those in the recovery phase. Only IL-12p70 and MCP-2 showed a five-fold increase in the HFMD patients in the recovery phase compared with the controls.

The cytokine (IL-1 β , IL-8, IP-10, IL-12p40, IL-12p70, TNF- α , RANTES, and MIP-1 α) concentrations in the cerebrospinal fluid were detected using Milliplex beads. Higher levels of IL-8, IP-10, and RANTES were observed in the acute-HFMD patients compared with those in the recovery phase (**Figure 2B**). The MIP-1 α levels of acute HFMD patients were higher than those in the controls, but showed no significant changes compared with those in the recovery phase (**Figure 2C**). The IL-1 β , IL-12p70, IL-12p40, and TNF- α concentrations in the cerebrospinal fluid were below the detection level by the Milliplex beads assay. All results confirmed that inflammatory cytokines are involved in virus-infected encephalitis.

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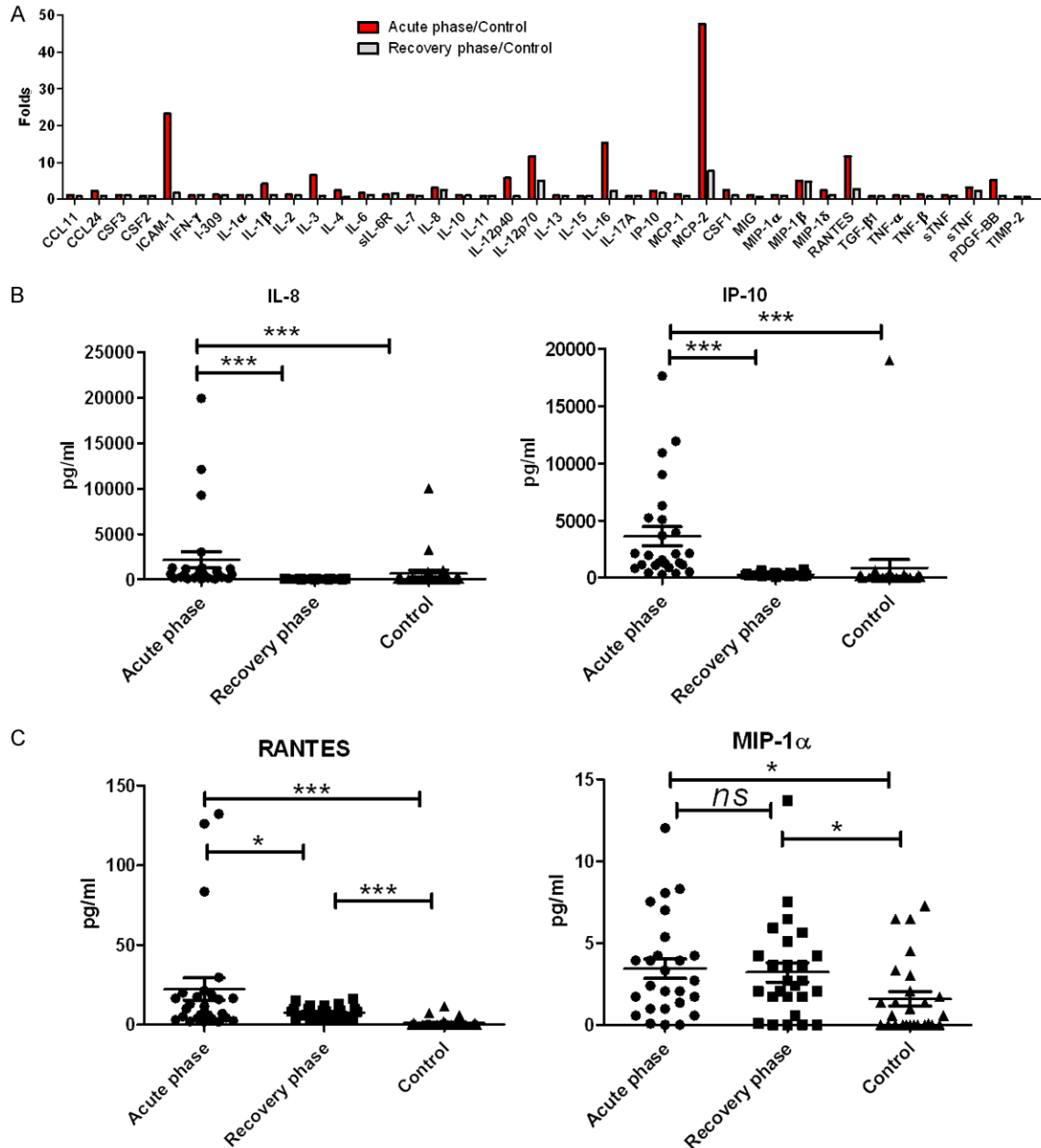


Figure 2. Detection of cytokines in cerebrospinal fluids from severe HFMD patients in the acute and recovery phases. (A) Cytokine levels measured using the Raybiotec cytokine antibody array (n = 4). IL-8 and IP-10 (B) RANTES and MIP-1α (C) detected using Milliplex beads (n = 26). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Comparison of IL-8 and IP-10 levels in serum and cerebrospinal fluid

We detected IL-8 and IP-10 concentrations in both sera and cerebrospinal fluids from severe HFMD patients in the acute phase. The IL-8 level was significantly higher in the cerebrospinal fluid at approximately 10-1000 folds (mean: 30 folds) compared with that in sera. Moreover, the IP-10 level in the cerebrospinal fluid was increased by approximately 2-125 times (mean:

30 folds), as shown in **Figure 3A**. Moreover, the serum IL-8 and IP-10 levels were not correlated with their levels in the cerebrospinal fluid (**Figure 3B**), indicating that IL-8 and IP-10 levels are highly dependent on the inflammatory state of the nervous system.

Discussion

Severe HFMD with nervous system involvement is the main cause of child deaths. Here we

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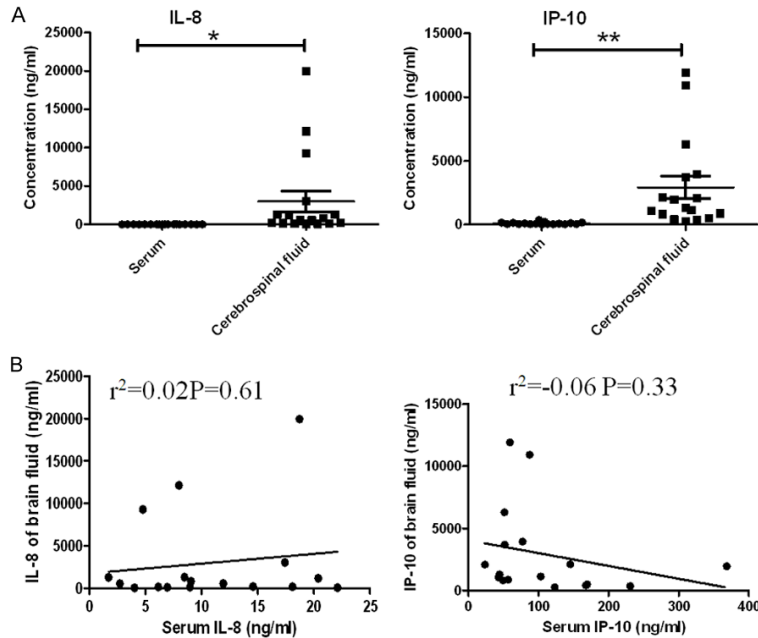


Figure 3. Comparison of IL-8 and IP-10 in the sera and cerebrospinal fluid from severe HFMD patients. A. Comparison of IL-8 and IP-10 in the sera and cerebrospinal fluid using paired Student's t test. B. Linear regression of serum IL-8 and IP-10 levels with their cerebrospinal fluid concentrations. *, $P < 0.05$; **, $P < 0.01$.

show that numerous serum cytokines, including CCL24, CSF-2, IFN- γ , I-309, IL-1 β , IL-4, IL-2, IL-11, IL-12p70, IL-12p40, IL-15, IL-16, IL-17A, MCP-2, CSF-1, MIG, and TNF- α , are involved in severe HFMD. Moreover, ICAM-1, IL-3, IL-8, IP-10, IL-12p40, IL-12p70, IL-16, MCP-2, RANTES, MIP-1 β and PDGF-BB concentrations in the cerebrospinal fluids are enhanced in the acute phase of the disease. Severe HFMD patients is then associated with the inflammatory-cytokine storm.

IL-8, which mediates the function of neutrophil recruitment, is the most observed inflammation cytokine [13]. Severe cases present a significantly higher frequency of the IL8-251 AT and TT genotypes than in mild cases [14]. IP-10 (IFN- γ inducible protein-10) promotes chemokine receptor CXCR3 expression on T and NK cells [15]. RANTES stimulates T lymphocytes to migrate into inflammation sites [16]. Serum IL-8 and IP-10 are enhanced in common HFMD patients but decreased in severe HFMD patients. IL-8, RANTES and IP-10 in the cerebrospinal fluid were sharply enhanced in the acute phase of severe HFMD patients. Thus, serum IL-8 and IP-10 levels are not good markers for

diagnosing severe HFMD. Moreover, high IL-8, RANTES and IP-10 levels in the cerebrospinal fluid are definite markers for severe HFMD.

IL-1 β , IL-6 and TNF- α are important inflammation mediators [17]. Serum IL-1 β is increased in HFMD patients but showed no changes in severe and common cases. However, the IL-1 β in the cerebrospinal fluid exhibited no significant change in severe HFMD patients. Cerebrospinal fluid IL-1 β could not be detected using the Milliplex method. Furthermore, serum and cerebrospinal fluid IL-6 showed no significant changes. Serum TNF- α is enhanced in severe HFMD patients; however, cerebrospinal fluid TNF- α displayed no changes. Therefore, cerebrospinal fluid levels of IL-1 β , IL-6 or TNF- α may not be

crucial in the differential diagnosis of severe HFMD.

As Th1 cell-associated cytokines, IL-12p40 and IL-15 are increased particularly in the sera of CNS-complicated HFMD patients [18]. The serum IL-8 and IP-10 levels would decrease in severe HFMD patients; therefore, variations in Th1 cell-associated cytokines in the sera are highly valuable biomarkers for diagnosing severe HFMD. As a Th17 cytokine, IL-17A plays a pathogenic role in CNS-related inflammation [19]. The IL-17F 7488C allele is associated with protection against encephalitis in Chinese patients with EV71-related HFMD [20]. However, IL-17A exhibited no significant changes in common and severe HFMD patients in this work. More samples would be used to clarify the discrepancies. Moreover, no significant changes in IL-4 and IL-13, which are Th2-associated cytokines [21], were observed in HFMD patients of different severities. These results indicated that HFMD inflammation with encephalitis is highly likely to be associated with Th1 cells.

The cerebrospinal fluid generally exhibits different biological characteristics compared with

the blood because of the blood-brain barrier [22]. For CNS-complicated HFMD patients, cerebrospinal fluid IL-8 and IP-10 levels are sharply 30-fold higher on average compared with the serum levels. The IL-8 and IP-10 serum levels are not correlated with the corresponding cerebrospinal fluid level. Thus, the physician should measure the IL-8 and IP-10 levels in cerebrospinal fluids, which directly reflects inflammation in the CNS. Furthermore, serum IL-8 and IP-10 levels should not be used as the differential diagnosis markers for severe and common HFMD.

In summary, severe HFMD patients exhibit inflammatory cytokine storms both in the sera and cerebrospinal fluid. IL-8, IP-10 and RANTES levels in cerebrospinal fluids could be used as biomarkers for diagnosing severe HFMD, whereas increased serum levels of IL-12p40 and IL-15 could be used as predictive factors for severe HFMD.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ying Hua, Department of Neurology, Wuxi Children's Hospital, 299 Qingyang Road, Wuxi 214023, Jiangsu Province, China. Tel: 86-510-85350511; E-mail: huayingwxey@medmail.com.cn; Dr. Weijuan Gong, Department of Immunology, School of Medicine, Yangzhou University, 11 Huaihai Road, Yangzhou 225009, Jiangsu Province, China. Tel: 86-514-87978820; Fax: 86-514-87341733; E-mail: wjgong@yzu.edu.cn

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